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DESCRIPTION

GLUCOSE DEHYDROGENASE/CYTOCHROME FUSION PROTEIN

Technical Field

The present invention relates to a fusion protein of a glucose dehydrogenase and a cytochrome, and a glucose assay using the same.

Background Art

Blood glucose level is an extremely crucial indicator in clinical diagnosis as an important marker for diabetes. In addition, in fermentative production using microorganisms, determination of glucose concentration is an important parameter for process monitoring. A glucose dehydrogenase having pyrroloquinoline quinone as a coenzyme (PQQGDH) has a high oxidation activity for glucose and it does not require oxygen as an electron acceptor because PQQGDH is a coenzyme-linked enzyme. Thus PQQGDH is an promising enzyme to be applied to assay techniques, for example, as a sensing element of a glucose sensor.

In order to assay glucose by an enzyme electrode having PQQGDH immobilized on its surface, it is necessary to add an electron mediator to the assay system for transferring electrons from PQQ, which is a redox center of PQQGDH, to the electrode. Accordingly, there are disadvantages in that the

performance of the electrode is limited depending on the stability or solubility of the electron mediator, and the background for measurement is increased by the reaction of a contaminant with the electron mediator. Furthermore, as the electron mediator is not suitable for use in vivo, the application of PQQGDH to a glucose sensor implanted in the body has been limited. In order to solve these problems, a method of immobilizing PQQGDH on an electrode with an electron transfer protein has been proposed (WO 02/073181). In this method, however, it is necessary to use extremely excess moles of electron transfer protein, which may lead to a cost problem. Thus, there has been a demand in the art for a sensing device of a "direct electron transfer-type" glucose sensor which requires no electron mediators.

Prior art documents related to the present invention includes: J. Okuda, J. Wakai, N. Yuhashi, K. Sode, Biosensors & Bioelectronics 18 (2003) 699-704; J. Okuda, J. Wakai, K. Sode, Anal. Lett. 35 (2002) 1465-1478.

Disclosure of the invention

An object of the present invention is to provide a modified PQQGDH which allows the construction of a direct electron transfer-type glucose sensor without involving the use of electron mediators.

The present invention provides a fusion protein of

pyrroloquinoline quinone glucose dehydrogenase (PQQGDH) and a cytochrome. PQQGDH is preferably a water-soluble PQQGDH derived from *Acinetobacter calcoaceticus*.

In the fusion protein of the present invention, the cytochrome has preferably been fused at the C-terminal side of PQQGDH. The cytochrome is preferably cytochrome c or cytochrome B562. Particularly preferably, the cytochrome is derived from a quinohemoprotein, which is a protein having both PQQ and a heme in one molecule. The cytochrome is preferably derived from a quinohemoprotein alcohol dehydrogenase. Particularly preferably, the cytochrome is derived from the electron transfer domain of quinohemoprotein ethanol dehydrogenase from *Comamonas testosteroni*.

Preferably the fusion protein of the present invention is either (a) or (b):

(a) a protein comprising an amino acid sequence represented by SEQ ID NO: 2;

(b) a protein comprising an amino acid sequence in which one or more amino acid residues have been deleted, substituted or added in the amino acid sequence (a) and having a glucose dehydrogenase activity and an electron transfer ability.

In another aspect, the present invention provides a gene encoding the fusion protein of the present invention, a vector and a transformant containing the gene. Preferably, the gene encoding the fusion protein has been integrated into the main

chromosome of the transformant.

In still another aspect, the present invention provides an enzyme electrode comprising the fusion protein of the present invention attached to the electrode, as well as a glucose sensor utilizing such an enzyme electrode.

The present invention further provides a method of measuring the glucose concentration in a sample comprising the steps of:

contacting the sample with the enzyme electrode of the invention; and

measuring electrons generated from the oxidation of glucose.

The use of the fusion protein of the present invention allows the construction of a direct electron transfer-type glucose sensor which requires no electron mediators.

Brief Description of the Drawings

Fig. 1 shows an example of the structure of a fusion protein.

Fig. 2 shows the sequence of a gene encoding the fusion protein shown in Fig. 1.

Fig. 3 shows the results of measuring the glucose concentration with the glucose sensor of the present invention in the presence of an electron acceptor.

Fig. 4 shows the response current of the glucose sensor

of the present invention in the absence of an electron acceptor.

Fig. 5 shows the results of measuring the glucose concentration with the glucose sensor of the present invention in the absence of an electron acceptor.

Fig. 6 shows an example of the structure of the flow cell portion of a flow-type glucose sensor.

Fig. 7 shows the response current of a flow cell-type glucose sensor utilizing a fusion protein of the present invention.

Fig. 8 shows the results of continuous running of a flow cell-type glucose sensor utilizing a fusion protein of the present invention.

Detailed Description of the Invention

Structure of fusion protein

A fusion protein of the present invention has a structure where PQQGDH and a cytochrome are bound to each other, and optionally has a linker region present between them.

PQQGDH is a glucose dehydrogenase having pyrroloquinoline quinone as a coenzyme, and catalyzes a reaction of oxidizing glucose to produce gluconolactone. Two types of PQQGDH are known: a membrane-bound enzyme and a water-soluble enzyme. The membrane-bound PQQGDH is a single-peptide protein having a molecular weight of about 87 kDa and is widely found in various gram-negative bacteria. On

the other hand, the water-soluble PQQGDH has been identified in several strains of *Acinetobacter calcoaceticus* (Biosci. Biotech. Biochem. (1995), 59 (8), 1548-1555), and the structural gene was cloned and the amino acid sequence thereof has been revealed (Mol. Gen. Genet. (1989), 217:430-436). Either of these PQQGDHs can be used in the present invention.

In the amino acid sequence of PQQGDH, part of the amino acid residues may be deleted or substituted, or another amino acid residue may be added. It has been revealed that the substitution of an amino acid residue in a specific region with another amino acid residue can improve the thermal stability of the enzyme or the affinity for a substrate while maintaining the activity of the enzyme of the oxidation of glucose (see, for example, JP-A-2000-350588, JP-A-2001-197888, JP-A-2000-312588). Such a modified PQQGDH may also be used in the fusion protein of the present invention.

The cytochrome is a heme protein having a function as an electron mediator, where one or more heme irons are covalently or non-covalently bound to a protein molecule. Many types of cytochromes including cytochrome b and cytochrome c have been isolated and identified in various organisms, and any of these cytochromes can be used in the present invention. Examples thereof include cytochrome b562 derived from bacteria such as *E. coli* strain B (Eur. J. Biochem. 202(2), 309-313 (1991)), *E. coli* strain K (Tower, M. K., Biochem. Biophys. Acta.

1143, 109-111 (1993)), *Acinetobacter calcoaceticus*, *Klebsiella pneumoniae*, *S. typhi*, *S. typhimurium*, *K. pneumoniae*, *Y. pestis*, *P. multocida* and *S. pneumoniae*. A chimeric protein produced from such a cytochrome may also be used.

Oxidoreductases having an electron transfer subunit or a heme-containing domain are known in the art. The heme protein subunit or the heme protein domain of such an enzyme is also included in the cytochrome in the present invention. In particular, the term cytochrome as used herein also includes the cytochrome domain of a quinoxinoprotein, a protein having PQQ as a coenzyme, which comprises a cytochrome with PQQ and a heme iron bound to a protein molecule. Also included is a cytochrome domain of quinoxinoprotein alcohol dehydrogenase, which is a quinoxinoprotein having an alcohol dehydrogenase activity. Examples of such an oxidoreductase include ethanol dehydrogenase, oligosaccharide dehydrogenase and the like.

Particularly preferably, a cytochrome c domain of quinoxinoprotein ethanol dehydrogenase (QHEDH) from *Comamonas testosteroni* is used in the invention. Recently the 3D structure of QHEDH was reported (J. Biol. Chem., 277, 2002, 3727-3732). QHEDH is composed of two different domains. A first domain is a PQQ-containing catalytic domain which is composed of an eight-bladed β -propeller structure similar to PQQGDH. A second domain positioned at the C-terminal region is a cytochrome c domain. These domains are separated by a

peptide linker region. In the QHEDH enzyme, an electron is transferred from PQQ, which is a redox center, to a respiratory chain via the cytochrome c, which is an electron acceptor.

In addition, the cytochrome to be used in the present invention may be a modified cytochrome in which part of the structure of a naturally occurring cytochrome has been modified. Such a modified cytochrome can be produced by substituting one or more amino acid residues of a naturally occurring cytochrome with another amino acid residue, which may or may not be a naturally occurring amino acid, or by deleting or adding one or more amino acids.

The linker region is a region that links PQQGDH to the cytochrome within the fusion protein. The linker region has a function to locate PQQGDH and the cytochrome in such a manner that the GDH activity is exerted and an electron can be efficiently transferred from PQQ to the cytochrome. The sequence of the linker region may be of a given naturally occurring or artificial amino acid sequence. For example, it may be an appropriate sequence derived from PQQGDH or the cytochrome, or may be a sequence derived from a vector used for constructing a gene encoding the fusion protein.

Method of producing fusion protein

The fusion protein of the present invention can be produced by ligating in-frame a gene encoding PQQGDH and a gene

encoding the cytochrome, optionally via the linker region, and allowing it to be expressed recombinantly. Fig. 1 shows one example of the fusion protein of the present invention. Fig. 2 shows the gene sequence encoding this fusion protein, where the sequence encoding PQQGDH, the linker region and the sequence encoding the cytochrome are linked to one another from the 5' side to the 3' side. The gene sequence encoding a naturally occurring water-soluble PQQGDH derived from *Acinetobacter calcoaceticus* is disclosed in Mol. Gen. Genet. (1989), 217: 430-436, and the gene sequence encoding the quinohemoprotein ethanol dehydrogenase (QHEDH) from *Comamonastes testosteroni* is disclosed in J. Biol. Chem., 277, 2002, 3727-3732. Based on these sequences, the gene encoding the fusion protein can be constructed through conventional gene manipulation techniques. Various techniques for gene manipulation are well known in the art.

The gene encoding the fusion protein obtained in this manner is inserted into an expression vector (for example, a plasmid), and the vector is transformed into an appropriate host (for example, *E. coli*). A large number of vector/host systems for expressing a heterologous protein are known in the art. The host may includes various organisms such as bacteria, yeast, and cultured cells.

A transformant expressing the fusion protein is cultured and the cells are collected by centrifugation or other means

from the culture medium and then disrupted by a French press or other means. The disrupted cells are centrifuged and a water-soluble fraction containing the fusion protein can be obtained. Alternatively, the expressed fusion protein can also be secreted into the culture medium by utilizing an appropriate host/vector system. The obtained water-soluble fraction is purified by ion-exchange chromatography, affinity chromatography, HPLC or other means, whereby the fusion protein of the present invention is prepared.

Enzyme electrode

The present invention also provides an enzyme electrode having the fusion protein according to the present invention immobilized on the electrode. The enzyme electrode is an electrode in which an enzyme has been immobilized on the surface of the electrode, such as a gold electrode, a platinum electrode or a carbon electrode. The enzyme electrode is broadly used in a biosensor that utilizes the reaction specificity of an enzyme for detecting any of a variety of biologically active substances in a specific manner.

The fusion protein of the present invention attached to the enzyme electrode may recognize the presence of a test subject (for example, glucose), catalyze the redox reaction and transfer the electron generated from the reaction to the electrode.

To construct the enzyme electrode, the fusion protein of the present invention is immobilized on an electrode. Immobilization methods include, for example, a method using a crosslinking reagent, a method of entrapping the enzyme into a macromolecular matrix, a method of coating the enzyme with a dialysis membrane, a method of immobilizing the enzyme in a polymer such as a photo-crosslinking polymer, an electric conductive polymer or a redox polymer. Combinations of these methods may also be used. Typically, the fusion protein of the present invention is immobilized on a carbon electrode with glutaraldehyde, and then unreacted glutaraldehyde is blocked by a treatment with a reagent having amine groups.

Glucose sensor

In another aspect, the present invention provides a sensor comprising the enzyme electrode of the present invention as a working electrode. The sensor as used herein means an assay system for electrochemically measuring the concentration of an objective test substance, and includes three electrodes, a working electrode (the enzyme electrode), a counter electrode (such as a platinum electrode) and a reference electrode (such as an Ag/AgCl electrode). Alternatively, it may be a two-electrode system composed of a working electrode and a counter electrode as is used in a conventional simple blood glucose monitoring system. The

sensor may further comprise a constant temperature cell that holds a buffer solution and a test sample, a power source to apply voltage to the working electrode, an ampere meter, and a recorder. The sensor may be of a batch type or a flow type. In particular, the sensor is a flow-type sensor capable of continuously measuring blood glucose level, where the two-electrode system or the three-electrode system having the enzyme of the present invention immobilized thereon is inserted into a flow of continuously supplied blood sample or dialyzed sample, or into a blood sample or an interstitial fluid sample. The structure of such an enzyme sensor is well known in the art, and is described in, for example, Biosensors-Fundamental and Applications-Anthony P. F. Tuner, Isao Karube and George S. Wilson, Oxford University Press 1987.

Figure 6 shows the schematic view of a flow cell portion of a preferred flow-type sensor of the present invention. A flow channel allowing a sample to flow at a predetermined rate is provided in a flow cell 1. A test substance diluted with an appropriate buffer solution is applied to the cell through a sample inlet port 20, and discharged from a sample outlet port 22 and directed to a sample drain. The flow cell 1 is equipped with a working electrode 10, a counter electrode 12, and a reference electrode 14, where the enzyme electrode of the present invention is used as the working electrode 10. A constant voltage is applied to the working electrode 10 from

a potentiostat (not shown). While two working electrodes are shown in Fig. 6, one working electrode system may be applied.

Assay of glucose

An assay of the glucose concentration using the glucose sensor of the present invention can be carried out as follows. A buffer solution is placed in the constant temperature cell of the sensor and a constant temperature is maintained. An enzyme electrode carrying the fusion protein of the present invention is used as the working electrode, and, for example, a platinum electrode and an Ag/AgCl electrode are used as the counter electrode and the reference electrode, respectively. A constant voltage is applied to the working electrode, and after the current reaches a steady state, a sample containing glucose is added to the constant temperature cell, and then the increase in the current is measured. The glucose concentration in the sample can be calculated according to a calibration curve generated with standard glucose solutions of known concentrations.

The entire contents of all the patents and reference documents explicitly cited in the present specification are incorporated herein by reference. In addition, the entire contents described in the specification and drawings of Japanese Patent Application No. 2003-340092, on the basis of which the present application claims a priority, are also

incorporated herein by reference.

Examples

The present invention will be described in more detail with reference to the examples below, however, these examples are not to be construed to limit the scope of the invention.

Construction of expression vector

The structural gene of PQQGDH (without a stop codon) and the electron transfer domain of QHEDH were amplified by the PCR method from the genomes of *A. calcoaceticus* LMD 79.41 and *C. testosteroni* ATCC 15667, respectively, using primers containing a restriction enzyme recognition site at the 5'-terminus. The primers used are as follows.

gdhB; sense

5'-GGCCATGGATAAACATTTATTGGCTAAAATTGCTTTAT-3' (SEQ ID NO: 3)

antisense

5'-GGGGGAGCTCCTTAGCCTTATAGGTGAAC-3' (SEQ ID NO: 4)

qhcdhcytc domain; sense

5'-GGGGGAGCTCGGCAAGGCCAGGATGCCGGA-3' (SEQ ID NO: 5)

antisense

5'-GGGGAAGCTTTCAGGGCTTGGGCCGGATGG-3' (SEQ ID NO: 6)

These PCR products were inserted into the multicloning site of an expression vector, pTrc99A (Amersham Biosciences, Sweden) to prepare an expression vector pGBET. In this way,

a fusion gene was constructed in which the cytochrome c domain of QHEDH was linked to the C-terminal side of PQQGDH via a linker region (Fig. 2). The sequence encoding PQQGDH is indicated by upper case letters and the sequence encoding the cytochrome is indicated by lower case letters. The restriction enzyme recognition site is shown by double underline and the linker region is shown by underline. The linker region between PQQGDH and the cytochrome c domain is composed of 24 amino acid residues derived from the native QHEDH.

The expression of ccm gene is essential for expressing the heme-containing cytochrome c in *E. coli*. Therefore, ccm is constitutively expressed by using a plasmid pEC86 (kindly gifted by Professor Linda Toeny-Meyer, ETH Switzerland) which contains the ccm gene necessary to mature the cytochrome c under the control of Km promoter.

E. coli DH5 α was transformed with pGBET and pEC86. A transformant containing both the fusion protein expression vector and the ccm expression vector turned pink, suggesting that the mature cytochrome c is produced in the cell.

Expression and purification of fusion protein

The transformant was cultured in MMI medium at 30°C for 10 hours under semi-aerobic conditions, and the cells were collected and resuspended in a 10 mM potassium phosphate buffer solution (pH 7.0). The suspended cells were disrupted by a

French press (110 MPa), ultracentrifuged (160,500 x g, 1.5 h, 4°C), and then the supernatant was dialyzed with a 10 mM potassium phosphate buffer solution (pH 7.0). The obtained supernatant was applied to a Resource S cation-exchange column (Amersham Biosciences) which had been equilibrated with a 10 mM potassium phosphate buffer solution (pH 7.0), and the enzyme was eluted with a gradient of NaCl (5 to 150 mM) in a 10 mM potassium phosphate buffer solution (pH 7.0).

The purified enzyme showed a single band at a position of about 65 kDa on SDS-PAGE, which corresponds to the molecular weight predicted for the fusion protein. This band was also stained by heme staining.

The UV/Vis spectrum of the purified fusion protein showed the peak of the oxidized type of cytochrome c at a wavelength of 411 nm. When sodium hydrosulfite (a reducing agent) was added, the fusion protein was reduced and showed peaks at 416 nm, 522 nm and 551 nm, which are typical for the reduced type of cytochrome c. From these results, it was confirmed that the fusion protein has a heme and functions as cytochrome c.

Next, in order to examine the intramolecular electron transfer between PQQ and the cytochrome c domain, glucose was added to the oxidized form of the fusion protein. When 20 mM glucose was added in the absence of an electron acceptor, the spectrum of cytochrome c changed from the oxidized form to the reduced form with time. These results indicate that the fusion

protein had GDH activity, and that intramolecular electron transfer from a redox center PQQ to cytochrome c occurred in association with the oxidation of glucose.

Measurement of enzyme activity

The measurement of the enzyme activity was carried out in a 10 mM MOPS-NaOH buffer solution (pH 7.0) containing 0.06 mM DCIP and 0.6 mM PMS. The enzyme activity for oxidizing 1 μ mol of glucose in 1 minute was defined as 1 unit. The dynamic parameters of the purified enzyme are shown in Table 1.

	Km (mM)	Vmax (U/mg)	V max/Km
Glucose	23	3057	133
Maltose	15	1721	114
Lactose	19	1584	82

The fusion protein of the present invention has GDH activity of about 3000 U/mg of protein, which corresponds to about 70% of the activity of the wild-type PQQGDH. Furthermore, the Km value and the substrate specificity of the fusion protein for glucose were substantially the same as those reported for the wild type enzyme (Biocatal. Biotransform. 20, (2002), 405-412).

Measurement of glucose concentration in the presence of electron acceptor

The fusion protein of the present invention(250 units;

about 100 μ g) or 350 U of the wild-type PQQGDH was immobilized on a glassy carbon electrode by exposing to glutaraldehyde vapor. The electrode was immersed in a 20 mM MOPS buffer solution (pH 7.0) containing 1 mM CaCl_2 , and the response to glucose was measured at an applied voltage of +400 mV (vs. Ag/AgCl) in the presence of 10 mM potassium ferricyanide as an electron acceptor. The results are shown in Fig. 3, where "QH-GDH" indicates the fusion protein produced in this Example, and "GB WT" indicates the wild-type PPQGDH.

Under the above conditions, only a very low response was observed in the electrode on which the wild-type PQQGDH had been immobilized. In particular, the reaction with the electron acceptor was the rate-limiting step at a glucose concentration of 0.2 mM or higher, and the response was saturated. On the contrary, a very high response was obtained in the electrode on which the fusion protein of the present invention had been immobilized. In other words, glucose concentration-dependent response was observed even at a glucose concentration at which the response was saturated in the electrode having the wild-type enzyme. A good response was also exhibited at 5 to 10 mM or higher concentrations, which represent the glucose level in the human blood. These results demonstrated that the fusion protein of the present invention could give a higher response than the wild-type enzyme in the presence of an electron acceptor.

Measurement of glucose concentration in the absence of electron acceptor

Next, the ability of the fusion protein to transfer an electron to an electrode was examined in the absence of an electron acceptor, with comparing to a mixture of the wild-type enzyme and an electron transfer protein. A 20 mM MOPS buffer solution (pH 7.0) containing 500 U of QH-GDH was mixed with carbon paste (0.5 g of graphite powder and 0.3 ml of a liquid paraffin, BAS Inc., West Lafayette, USA) and the mixture was lyophilized. The wild-type PQQGDH and an equivalent molar amount of cytb562, or an equivalent mass of BSA (bovine serum albumin) to cytb562 were used as a control. Then, the lyophilized mixture was applied to the tip of a carbon paste electrode (a diameter of 3 mm, BAS Inc.). The electrode was stored at 4°C in a 20 mM MOPS buffer solution (pH 7.0) until use. The measurement was carried out at 25°C in a 20 mM MOPS buffer solution (pH 7.0) containing 1 mM CaCl_2 . An increase in the current value with the addition of glucose was detected under an applied voltage of +300 mV vs. Ag/AgCl.

The enzyme electrode on which the fusion protein of the present invention had been immobilized showed a prompt response to the addition of glucose, and the sensor signal reached a steady current within 10 seconds after the addition of glucose (Fig. 4). In Figure 4, arrows indicate the addition of glucose.

On the contrary, no increase in the current was observed in the control electrode on which the wild-type PQQGDH and cytb562 or BSA had been immobilized.

Assay of glucose

A calibration curve for the sensor of the present invention was created using glucose solutions of various known concentrations (Fig. 5). In Figure 5, "QH-GDH" indicates the fusion protein produced in this Example, and "GB wt" indicates the wild-type PQQGDH as a control. The observed increase in the current was proportional to the glucose concentration in the concentration range from 0.01 mM (i.e., minimum detectable concentration) to 5 mM. Furthermore, the current response was dependent on the amount of the enzyme. The sensitivity of the sensor was $9.65 \mu\text{A M}^{-1}\text{cm}^{-2}$.

Continuous measurement of glucose

The glucose level was continuously measured using an enzyme electrode carrying the fusion protein of the present invention. This experiment was performed for the purpose of applying the fusion enzyme of the present invention to a continuous measurement-type blood glucose measurement system that has attracted attention in recent years. The system used in this experiment was assembled in accordance with the configuration of a conventional continuous measurement-type

blood glucose measurement system.

The measurement was carried out using a flow cell shown in Fig. 6. A flow channel for flowing a sample is provided in a flow cell 1. A test substance diluted with an appropriate buffer solution was applied to the cell through a sample inlet port 20, and discharged through a sample outlet port 22 into a sample drain. The flow cell 1 is equipped with two working electrodes 10, a counter electrode 12, and a reference electrode 14. A constant voltage was applied to the working electrode 10 from a potentiostat (not shown). The fusion protein (750 units) was mixed with carbon paste and crosslinked to each other with glutaraldehyde and then, immobilized on the surface of the working electrode. A 100 mM potassium phosphate buffer solution (pH 7.0) containing 1 mM CaCl_2 was continuously supplied to the flow cell. The applied voltage was set to +300 mV vs. Ag/AgCl, and the flow rate was set to 0.5 ml/min. A sample solution with continuously variable glucose concentration was prepared by adding a solution containing 450 mg/ml glucose to the buffer solution at a given mixing ratio. The sample solution was continuously supplied to the flow cell with the enzyme electrode, and the response current was measured. The results are shown in Fig. 7. As shown in the figure, it was demonstrated that the glucose concentration could be continuously measured with the sensor based on the fusion protein of the present invention involving the direct

electron transfer within the sensor, i.e., without adding an electron acceptor.

Next, the long-term continuous running of the continuous glucose measurement system was investigated. When a sample containing 1 mM glucose was continuously supplied to the flow cell at a flow rate of 0.1 ml/min, a stable response was observed, and even after 72 hours, a response which was 70% or higher of the initial response value was observed (Fig. 8). These results showed that the continuous glucose measurement system utilizing the fusion protein of the present invention could be continuously operated for 3 days or longer, demonstrating that the fusion enzyme of the present invention will have a sufficient effectiveness for use in a continuous measurement-type blood glucose measurement system that has attracted attention in recent years.

Industrial Applicability

The fusion protein of the present invention and the enzyme electrode and the biosensor using the same are useful as a direct electron transfer-type glucose sensor for measuring blood glucose level.